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USAMRMC ltr, 5 Mar 2002

AD_____

AWARD NUMBER DAMD17-97-1-7244

TITLE: Gene Therapy of Disseminated Breast Cancer Using Adenoviral Vectors Targeted Through Immunological Methods

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE
Gene Therapy of Disseminated Breast Cancer Using Adenoviral Vectors Targeted Through Immunological Methods

5. FUNDING NUMBERS
DAMD17-97-1-7244

6. AUTHOR(S)
Rogers, Buck E., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Aug 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*
Targeting of adenovirus (Ad) vectors, encoding for therapeutic genes, to tumor-specific receptors on breast cancer cells should result in specific killing of these cells. Targeting is necessary to prevent gene transfer in normal tissues resulting from the wide array of normal cells by adenovirus. We have previously reported the use of an anti-knob antibody fragment (Fab), which prevents Ad infection, conjugated to folate to target adenovirus to folate receptor positive cells. In this report, the Fab has been conjugated to FGF2, EGF, and an anti-erbB-2 antibody to yield Fab-FGF2, Fab-EGF, and Fab-erbB2 conjugates, respectively. These conjugates were used to target an adenovirus encoding the firefly luciferase gene (AdCMVLuc) to FGF, EGF, and erbB-2 receptors on BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. Also, live-cell binding assays with radiolabeled Ad, FGF2, EGF, and anti-erbB-2 antibody were performed to determine relative receptor expression. These data suggest that there is a relationship between the level of receptor expression and gene transfer. Also, in many instances the targeted adenovirus yielded better gene transfer than adenovirus alone. In this regard, use of the Fab-FGF2 conjugate resulted in the best gene transfer in the BT-474, MDA-MB-134, and MDA-MB-231 cells, the Fab-EGF was best in the MDA-MB-468 and SK-BR-3 cells and the Fab-erbB-2 was best in the MDA-MB-453 cells. These results are significant in that they demonstrate that Ad vectors can be specifically delivered to breast cancer cells and result in enhanced gene transfer. This will be significant for treating disseminated breast cancer with Ad vectors.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer adenovirus, gene transfer, targeting, FGF2, EGF, erbB-2	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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B. R. Roy 8/28/98

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INTRODUCTION

Delivery of therapeutic genes to cancer cells *in vivo* using adenoviral vectors has been widely investigated because of their ability to accomplish *in vivo* gene transfer with high efficiency (1, 2, 3, 4). However, the broad tropism of the parent adenovirus causes the transduction of normal tissue *in vivo* (2) due to the wide expression of the receptor that binds the adenovirus fiber knob (5, 6, 7). This lack of tumor-specific targeting of adenovirus is its major drawback. Thus, it is necessary to ablate endogenous adenoviral tropism and introduce new tropism that is specific for the tumor site to accomplish tumor-specific gene transfer. The purpose of this research is to develop a method for the specific delivery of recombinant adenoviral vectors to disseminated breast cancer. The scope of the research will focus on targeting of adenoviral vectors to specific receptors found on breast neoplasms both *in vitro* and *in vivo.* This will be accomplished using adenoviral vectors encoding for both reporter genes and therapeutic genes.

Previous work has demonstrated that it is possible to target adenoviral vectors to heterologous cellular receptors for accomplishing efficient gene transfer. We have developed an immunological strategy to ablate endogenous adenovirus tropism and introduce novel adenovirus tropism through the use of an adenovirus anti-knob monolconal antibody conjugated to ligands specific for tumor cell receptors (8, 9, 10). Our initial studies utilized the Fab fragment of the anti-knob antibody conjugated to the vitamin folic acid (to yield a Fab-folate conjugate) for targeting adenovirus to folate receptor-positive cells *in vitro* (8). When KB cells, a folate receptor-positive human nasopharyngeal carcinoma cell line, were infected with an adenovirus encoding the firefly luciferase gene (AdCMVLuc), a high level of gene transfer was observed. In contrast, the unconjugated Fab fragment blocked AdCMVLuc infection resulting in a 99% inhibition of luciferase expression. When the AdCMVLuc was premixed with the Fab-folate conjugate, the level of luciferase expression was restored to ~80% of the level when AdCMVLuc alone was used. This demonstrated that the retargeted adenovirus was still capable of efficient gene transfer. Specificity was demonstrated by inhibiting the infection of the folate receptor targeted AdCMVLuc with an excess of free folate. The free folate saturated the target receptor, preventing the binding of the adenoviral complex. Thus, these studies demonstrated the feasibility to target adenovirus to heterologous cellular receptors through immunological methods.

We have also demonstrated that the Fab can be conjugated to the basic fibroblast growth factor (FGF2) for targeting adenoviral vectors to FGF receptors (9). Goldman *et al.* demonstrated that the luciferase expression in a panel of Kaposi's sarcoma (KS) cells was significantly increased when infected with AdCMVLuc premixed with a Fab-FGF2 conjugate compared to cells infected with AdCMVLuc alone (9). This is in contrast to infection of the KB cells with AdCMVLuc and the Fab-folate conjugate in which luciferase expression was restored to ~80% of the level with AdCMVLuc alone. The enhancement of gene expression in the KS cells is likely due to the fact that the KS cells are relatively resistant to adenovirus infection (implying that they have a low number of adenovirus receptors) so that targeting to FGF receptors (present in high levels on the KS cells) leads to an increase in luciferase

expression. In contrast, the KB cells are susceptible to AdCMVLuc infection and thus do not demonstrate an increase in luciferase expression when targeting using the Fab-folate conjugate. The KS cells were also infected with an adenovirus encoding the herpes simplex virus thymidine kinase gene (AdCMVHSVtk) or the AdCMVHSVtk mixed with the Fab-FGF2 conjugate. Cytotoxicity in the cells were assesed in the presence or absence of ganciclovir. The results show that targeting AdCMVHSVtk with Fab-FGF2 resulted in a significant enhancement of ganciclovir-mediated killing in the KS cells. Thus, targeting of adenovirus to heterologous cellular receptors can not only lead to cell-specific gene transfer, but it can enhance the level of gene transfer depending on the susceptibility of the cell to adenovirus infection and the amount of target receptor present.

The purpose of this proposal is to utilize this immunological method of targeting adenoviral vectors for specifically delivering adenoviral vectors to breast carcinomas. The use of adenoviral vectors will capitalize on the high efficiency of *in vivo* gene transfer for specific delivery of therapeutic genes to breast lesions. The specific aims of this proposal were 1.) to conjugate the anti-knob Fab antibody to ligands that have a high affinity for cell surface receptors overexpressed in breast cancer cells, 2.) to evaluate the efficiency and specificity of adenoviral-mediated gene transfer using these conjugates *in vitro* in breast cancer cell lines, and 3.) to evaluate the efficiency and specificity of adenoviral-mediated gene transfer using these conjugates *in vivo* in an animal model of breast cancer. In addition, studies have been undertaken to understand the relationship between gene transfer and the expression of the receptor being targeted. This report will summarize the progress made thus far in achieving these specific aims and discuss the results in relation to the Statement of Work outlined in the proposal.

Body of Work

Experimental Methods

Conjugation of FGF2, EGF, and anti-erbB-2 antibody to Fab. The anti-knob antibody (1D6.14) was generated against the adenovirus serotype 5 knob region as previously described and digested with papain to obtain the Fab fragment (8). The FGF2 was modified to contain only one cysteine (11) to be used in the conjugation to the Fab. The Fab-FGF2 conjugate (kindly provided by Barbara Sosnowski) was made as previously described (9). Briefly, the Fab was modified with N-succinimidyl-3-[2-pyridyldithio]propionate (SPDP) at a 1:3 molar ratio and incubated at room temperature for 30 min. Excess SPDP was removed by dialysis and the Fab-SPDP added to reduced FGF2 at a 1:2 molar ratio and incubated overnight at 4°C. The reaction mixture was then purified using a size-exclusion column and the Fab-FGF2 conjugate collected. The size and activity of the conjugate was analyzed by Western blot and ELISA assay (9).

For conjugation of EGF (Upstate Biotechnology, Lake Placid, NY) to Fab, both

EGF and Fab were modified with SPDP at 1:1.2 and 1:10 molar ratios, respectively, in a 0.1 M H_3BO_3, buffer at pH 8.5. The reactions were incubated at room temperature for 30 min then purified using a Bio-Spin 6 (Bio-Rad Laboratories, Hercules, CA) size-exclusion column. The Fab-SPDP was purified using a 0.1 M NH_4OAc pH 5.5 buffer, while the EGF-SPDP was purified using a 0.02 M Na_2HPO_4, 0.1 M NaCl, 1 mM EDTA pH 7.2 buffer. The Fab-SPDP was reduced with dithiothreitol (DTT) for 30 min at room temperature and purified using a Bio-Spin 6 size-exclusion column equilibrated in PBS buffer. This yields a free sulfhydryl group on the Fab that is reactive with the SPDP group on the EGF protein. The EGF and Fab were then mixed at a 5.5:1 molar ratio and incubated at 4°C for 16 h. The resulting Fab-EGF conjugate was purified using a Bio-Rad size-exclusion HPLC column. The presence of the conjugate was validated by mass spectrometry and stored at 4°C until use in adenoviral targeting experiments.

Conjugation of an anti-erbB-2 monoclonal antibody (kindly provided by Mansoor Saleh, University of Alabama at Birmingham) to the Fab was similar to the procedure described for the Fab-EGF conjugation. Briefly, both the Fab and anti-erbB-2 antibody were incubated with SPDP at a 1:10 molar ratio for 30 min at room temperature in 0.1 M H_3BO_3 pH 8.5. The erbB-2-SPDP was purified using a Bio-Spin 6 size-exclusion column equilibrated with 0.1 M NH_4OAc pH 5.5, while the Fab-SPDP was purified using a column equilibrated with 0.02 M Na_2HPO_4, 0.1 M NaCl, 1 mM EDTA pH 7.2. The erbB-2-SPDP was reduced with DTT for 30 min at room temperature and purified using a Bio-Spin 6 column equlibrated in PBS buffer. The reduced erbB-2-SPDP was then mixed with the Fab-SPDP in a 1:3 molar ratio and incubated at 4°C for 16 h. The resulting Fab-erbB-2 conjugate was purified using a Bio-Rad size-exclusion HPLC column. The conjugate was validated using adenoviral targeting experiments.

Cell lines and adenoviral vector. The breast carcinoma cell lines MDA-MB-134, MDA-MB-231, BT-474, MDA-MB-453, MDA-MB-468 and SK-BR-3 were obtained from the American Type Culture Collection (Rockville, MD). The BT-474 cells were maintained in RPMI 1640 with bovine insulin (10 μg/ml) and L-glutamine (300 μg/ml) with 10% fetal bovine serum (FBS); MDA-MB-134 were maintained in Leibovitz's L-15 medium with 20% FBS; MDA-MB-231, MDA-MB-453 and MDA-MB-468 were maintained in Leibovitz's L-15 medium with 10% FBS; and SK-BR-3 were maintained in McCoy's medium with 10% FBS. The AdCMVLuc, an E1-,E3-deleted Ad5 vector which expresses firefly luciferase driven by the CMV promoter, was generously provided by R.D. Gerard (University of Leuven, Leuven, Belgium).

Radiolabeled ligand binding assay and scatchard analysis. Binding assays were performed on the various breast cancer cell lines with radiolabeled FGF2, EGF, and anti-erbB-2 antibody to determine the relative level of receptor expression on these cell lines. Also, binding assays were performed with radiolabeled adenovirus (^{125}I-Ad; radiolabeled using the iodogen method by the Comprehensive Cancer Center Radiolabeling Core Facility) to determine relative levels of adenovirus receptor expression. Scatchard analysis was performed using radiolabeled EGF and anti-

erbB-2 antibody to quantitate the receptor expression on particular cell lines. Scatchard analysis using ^{125}I-FGF2 (DuPont NEN® Research Products, Boston, MA) are currently being performed. For the binding assays, cells were harvested using 4 mM EDTA, 0.05% KCl, 24 h after plating (~80% confluent). The cells were counted, pelleted, and resuspended in 0.1% BSA in PBS at 1 x 10^7 cells/ml. Approximately 0.05 nM of ^{125}I-FGF2, ^{125}I-EGF (Amersham Life Science, Arlington Heights, IL), or ^{125}I-anti-erbB-2 (radiolabeled using the iodogen method by the Comprehensive Cancer Center Radiolabeling Core Facility) was added to 1 x 10^6 cells with or without a 1000-fold molar excess of unlabeled ligand to block and incubated 1 h at 4°C. After dilution, the cells were pelleted and counted on a gamma counter (Packard Instrument Co., Downers Grove, IL). Scatchard analysis was performed as described above except the cells were resuspended at 2.5 x 10^6 cells/ml. Various amounts of unlabeled ligands (0.0001 nM to 1000 nM) were then added to 2.5 x 10^5 cells along with 0.05 nM of ^{125}I-EGF or ^{125}I-anti-erbB-2 and incubated 1 h at 4°C. After dilution, the cells were pelleted, counted on a gamma counter, and the results were analyzed using the radioligand program Kell for Windows (Biosoft, Ferguson, MD). Non-specific binding was considered to be the amount of radioactivity measured using the greatest amount of unlabeled ligand.

Targeting of AdCMVLuc in vitro. To assess AdCMVLuc gene transfer or AdCMVLuc gene transfer targeted with Fab-FGF2, Fab-EGF, or Fab-erbB-2, cells were seeded 24 h prior to infection in 12 well plates with 4.8 x 10^4 cells in triplicate. For Fab-EGF and Fab-erbB-2 targeting experiments, cells were blocked with 300 μl/well of recombinant Ad5 knob (20 μg/ml), EGF (6.7 μg/ml) or erbB-2 antibody (10 μg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugates. For Fab-FGF2 experiments, a neutralizing amount of Fab or a rabbit anti-FGF2 polyclonal antibody (Sigma Chemical Co., St. Louis, MO) was directly incubated with AdCMVLuc or AdCMVLuc-Fab-FGF2 conjugate respectively for 30 min at room temperature for blocking gene transfer. For targeting experiments, AdCMVLuc (4.5 x 10^{10} plaque forming units (pfu)/ml) were preincubated with either PBS or the optimal amount (as determined by titering the conjugate against AdCMVLuc; data not shown) of Fab-FGF2, Fab-EGF, or Fab-erbB-2 for 30 min at room temperature. The mixtures were diluted with OptiMEM (Gibco BRL, Grand Island, NY) to a final concentration of 4.8 x 10^6 pfu/ml and 200 μl added to each well (20 pfu/cell) and incubated for 1 h at 37°C. Cells were then washed with PBS, supplemented with complete media and incubated 24 h before lysis. Relative light units (RLU) were measured in a Berthold luminometer (Lumat LB 9501, Wallac, Gaithersburg, MD) using a luciferase assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Statistical analysis was performed on the binding assays and gene transfer assays using a student's t-test.

Results

Conjugation of FGF2, EGF, and anti-erbB-2 antibody to Fab. Following purification of the conjugates, they were assayed for protein concentration using a Bradford assay. This gave protein concentrations of 0.34 mg/ml, 0.51 mg/ml, and 0.71

mg/ml for the Fab-FGF2, Fab-EGF, and Fab-erbB-2 conjugates respectively. The Fab-FGF2 conjugate was validated by Western blot and ELISA assay, while the Fab-EGF conjugate was validated by mass spectrometry. The Fab-erbB-2 conjugate could not be evaluated by mass spectrometry due to lower sensitivity for detecting higher molecular weight proteins, and thus was simply validated using adenoviral targeting assays.

Radiolabeled ligand binding assay and scatchard analysis. ^{125}I-Ad live-cell binding assays were performed on BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 to determine the relative amount of adenovirus receptor expressed on these cells (**Figure 1**). This assay will also be performed using the MDA-MB-453 and SK-BR-3 cells. **Figure 1** shows 11.5%, 7.3%, 8.0%, and 7.7% binding of ^{125}I-Ad to BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells respectively. The binding of ^{125}I-Ad to the BT-474 cells was significantly greater than to the other cell lines ($p < 0.001$), while the binding between the other cell lines did not differ ($p > 0.4$). The binding to all cell lines was reduced to < 3.5% when an excess of Ad5 knob was added to the cells to block. Thus, binding of ^{125}I-Ad to the cells was specific and the level of adenovirus receptor expression on MDA-MB-468, MDA-MB-134, and MDA-MB-231 was similar while the expression on the BT-474 lines was greater.

The binding of ^{125}I-FGF2 to all six cell lines is shown in **Figure 2**. The binding was between 35% and 55% for all cell lines. However, blocking studies with an excess of unlabeled FGF2 demonstrated a high level of non-specific binding that was still 20% to 38%. This high non-specific binding is likely due to ^{125}I-FGF2 binding to low affinity heparan-sulfate proteoglycans present on most cells. The binding to these low affinity sites may undermine the specificity of the Fab-FGF2 conjugate.

The binding of ^{125}I-EGF to the breast cancer cells in shown in **Figure 3**. The MDA-MB-468 and SK-BR-3 cells showed the highest level of binding at 49.4% and 66.0% respectively. The BT-474 and MDA-MB-231 cells showed intermediate binding of 12.5% and 11.8% respectively, while the MDA-MB-134 and MDA-MB-453 cells showed low binding of < 3.5%. An excess of unlabeled EGF blocked binding to < 3.1% for all cell lines. Thus, specific binding of ^{125}I-EGF was demonstrated in the BT-474, MDA-MB-468, MDA-MB-231, and SK-BR-3 cells with the MDA-MB-468 and SK-BR-3 cells showing significantly greater binding than the BT-474 and MDA-MB-231 cells ($p < 0.02$).

Binding of the ^{125}I-anti-erbB-2 monoclonal antibody to all of the cell lines is shown in **Figure 4**. The BT-474 and SK-BR-3 cells demonstrated high binding of 54.8% and 53.8% respectively. The MDA-MB-453 cells demonstrated intermediate binding of 28.7% and the other cell lines all showed low binding of < 6.5%. Binding specificity was demonstrated in BT-474, SK-BR-3, and MDA-MB-453 cells using an excess of unlabeled anti-erbB-2 monclonal antibody as an inhibitor. Thus, BT-474, SK-BR-3, and MDA-MB-453 cells demonstrated significantly higher binding of ^{125}I-anti-erbB-2 antibody than the other three cell lines ($p < 0.0001$).

Scatchard assays were performed on cell lines that demonstrated specific binding of a particular radiolabeled ligand (**Table 1**). Analysis using ^{125}I-FGF2 have yielded confounding results thus far, possibly due to the binding of ^{125}I-FGF2 to low

affinity sites (heparan sulfate proteoglycans) and high affinity sites. We hope to have this problem resolved soon. Scatchard analysis on BT-474, MDA-MB-468, and MDA-MB-231 cells using ^{125}I-EGF showed 8.3 x 10^3, 7.5 x 10^5, and 4.0 x 10^4, EGF receptors/cell respectively. These results agree with the relative binding of ^{125}I-EGF to these cell lines (**Figure 3**). Scatchard analysis on BT-474 cells using ^{125}I-anti-erbB-2 antibody gave 6.6 x 10^5 erbB-2 receptors/cell. Scatchard analysis on SK-BR-3 and MDA-MB-453 cells with ^{125}I-erbB-2 and on SK-BR-3 cells with ^{125}I-EGF are currently being performed.

Targeting of AdCMVLuc in vitro. **Figure 5** shows targeting of AdCMVLuc to BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells using the Fab-FGF2 conjugate. Targeting of AdCMVLuc to MDA-MB-453 and SK-BR-3 cells using Fab-FGF2 is currently being performed. This figure shows that the luciferase expression in BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells infected with AdCMVLuc alone was 1.1 x 10^6, 2.5 x 10^5, 9.7 x 10^4, and 7.6 x 10^5 relative light units (RLU). Luciferase expression was inhibited >90% in all cell lines when the AdCMVLuc was neutralized with Fab. Infection with the Ad-Fab-FGF2 conjugate resulted in an increase in luciferase expression in all cell lines, which was shown to be specific by blocking with an anti-FGF2 polyclonal antibody. The ratio of targeted luciferase expression to non-targeted luciferase expression will be referred to as the targeting index (Ti). Thus, BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells have Ti values of 3.3, 3.7, 44.6, and 4.8 respectively. It can be noted that in general, if a cell line is resistant to Ad infection, it will have a higher Ti when targeting a heterologous receptor compared to a cell line that is easily transduced by Ad. Thus, the MDA-MB-134 cells have a Ti of 44.6 primarily due to the low level of luciferase expression when infected with AdCMVluc alone (9.7 x 10^4 RLU).

Targeting of AdCMVLuc with the Fab-EGF conjugate to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, and SK-BR-3 cells is shown in **Figure 6**. This shows that infection of these cells with AdCMVLuc alone can be inhibited > 95% by blocking the adenoviral receptors on the cells with Ad5 knob. Also, targeting of AdCMVLuc with Fab-EGF to MDA-MB-468, MDA-MB-231, and SK-BR-3 cells resulted in Ti values of 11.5, 1.3, and 1.9, respectively, and specificity was demonstrated by inhibiting luciferase expression > 65% with an excess of EGF. The relative Ti values of the MDA-MB-468 and MDA-MB-231 cells are in agreement with the level of ^{125}I-EGF binding to these cells (**Figure 3**). The SK-BR-3 cells may have been expected to have a Ti value similar to the MDA-MB-468 cells due the their high level of ^{125}I-EGF binding; however, they are very susceptible to adenovirus infection compared to the MDA-MB-468 cells, which accounts for the lower than expected Ti. The Ti for the BT-474 cells was 0.3 and targeting was not specific as an excess of EGF did not inhibit infection. These results do not agree with the ^{125}I-EGF binding data from **Figure 3** which indicate that it should be possible to target EGF receptors on these cells. Thus, this targeting experiment must be investigated further. The Ti for the MDA-MB-134 cells was 0.2. This result is expected as the MDA-MB-134 cells do not express the EGF receptor (**Figure 3**).

Targeting of AdCMVLuc with the Fab-erbB-2 conjugate to BT-474, MDA-MB-

231, MDA-MB-453, and SK-BR-3 cells is shown in **Figure 7**. This shows that infection of these cells with AdCMVLuc alone can be inhibited >95% by blocking the adenoviral receptors on the cells with Ad5 knob. AdCMVLuc targeting with Fab-erbB-2 was specific in MDA-MB-453 and SK-BR-3 cells as demonstrated by > 75% inhibition with an excess of anti-erbB-2 antibody. These cells had Ti values of 0.6 and 1.6 respectively. The BT-474 cells have a Ti value of 1.3; however, erbB-2 targeted gene transfer was not inhibited by an excess of erbB-2 antibody. This will need to be investigated further. Targeting to the erbB-2 negative MDA-MB-231 cells was not specific, which agrees with the ^{125}I-anti-erbB-2 binding assay (**Figure 4**).

Discussion

Ad vectors are currently being investigated in clinical gene therapy trials for treatment of various malignancies (12, 13). However, the widespread tropism of adenoviral vectors has limited their specificity by causing transduction of non-tumor sites *in vivo* (2). The purpose of this proposal is to develop immunologically modified adenoviral vectors for specifically targeting breast cancer cells *in vitro* and *in vivo*. The Fab-FGF2, Fab-EGF, and Fab-erbB-2 conjugates have been made for use in targeting adenoviral vectors to breast cancer cells. These conjugates were to be made within the first 9 months of the project as outlined in the Statement of Work. They have been functionally validated by targeting AdCMVLuc to breast cancer cells as opposed to the methods described in the Statement of Work. These *in vitro* targeting assays are shown in **Figures 5-7** and were not proposed to be completed until the second year according to the Statement of Work.

In addition, ^{125}I-Ad binding assays (**Figure 1**) and ^{125}I-ligand binding assays (**Figures 2-4**) not mentioned in the Statement of Work have been performed to determine the relative number of adenoviral receptors and ligand receptors respectively on each cell line. These assays were performed to gain an understanding of the relationship between gene transfer and the expression of the receptors that the adenovirus and targeted adenovirus are binding to (either the adenovirus receptor or the receptors for FGF, EGF, or erbB-2). Following administration of the Ad vector, three distinct, sequential steps are required for expression of the gene in target cells: 1) attachment of the Ad vector to specific receptors on the surface of the target cell; 2) internalization of the virus; and 3) transfer of the gene to the nucleus where it can be expressed. It has been shown that the knob domain of the Ad fiber protein is primarily responsible for attachment to the adenoviral receptor, the first step in infection (14, 15). Following attachment, the next step in Ad infection is internalization of the virion by receptor-mediated endocytosis (7, 16, 17). This process is mediated by the interaction of Arg-Gly-Asp (RGD) sequences in the penton base of the adenovirus with secondary cellular receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (18, 19, 20). Post-internalization, the virus is localized in endosomes which are acidified, releasing the virions into the cytosol, whereby the genome is translocated to the nucleus of the host cell. We hypothesized that a correlation exists between

receptor expression and gene transfer. Thus, radioligand binding assays and targeted gene transfer assays were performed to test this hypothesis. This also suggests that assays need to be performed to determine the relative expression of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on these cells. If the various cell lines express different levels of integrins, then there may not be a direct correlation between adenovirus receptor expression or ligand receptor expression and gene transfer.

Table 2 summarizes the data generated thus far. This shows that FGF2 scatchard analysis will be performed on all of the cell lines, EGF scatchard analysis will be performed on SK-BR-3 cells and erbB-2 scatchard analysis will be performed on MDA-MB-453 and SK-BR-3 cells. Targeting assays will be performed on MDA-MB-453 and SK-BR-3 cells using Fab-FGF2. In addition, ^{125}I-Ad binding assays to MDA-MB-453 and SK-BR-3 cells will be performed. Neither scatchard assays nor targeting assays will be performed on cell lines that did not show > 5% binding of a particular ligand, unless the cell line is used as a negative control. In addition, more sophisticated statistical analyses will be performed on all of the data to determine significant differences amongst the different assays.

The EGF scatchard analyses agree with the ^{125}I-EGF binding assays in that the MDA-MB-468 cells express a high level of EGFr and the BT-474 and MDA-MB-231 cells express a lower level of EGFr. The scatchard analyses with FGF2 and erbB-2 will be completed to compare to the respective binding assays.

Targeting assays performed with AdCMVLuc and the Fab-FGF2 conjugate show that the MDA-MB-134 cells have the largest Ti. This agrees with the ^{125}I-FGF2 binding data which shows that the MDA-MB-134 cells bind the most radiolabeled FGF2. Targeting assays with the Fab-EGF conjugate show that the MDA-MB-468 cells have the largest Ti, in agreement with the ^{125}I-EGF binding assays and scatchard assays. Also, the MDA-MB-134 cells have the lowest Ti which is expected due to the low amount of ^{125}I-EGF binding. The SK-BR-3 cells have the highest luciferase expression when infected with the Ad-Fab-EGF conjugate (in agreement with the ^{125}I-EGF binding assay); however, the Ti is not as large as for the MDA-MB-468 cells due to the high level of gene transfer in SK-BR-3 cells when infected with AdCMVLuc alone. The AdCMVLuc targeting to BT-474 cells with Fab-EGF was not specific which is a confounding result that needs further investigation. Finally, targeting of AdCMVLuc to BT-474 and SK-BR-3 with the Fab-erbB-2 conjugate resulted in Ti values of 1.3 and 1.6, respectively. However, the targeting to the BT-474 cells was not inhibited by excess erbB-2 antibody, indicating that this needs further investigation. The MDA-MB-453 cells have a Ti that is lower than the BT-474 and SK-BR-3 cells, agreeing with the ^{125}I-erbB-2 binding assay, although it might be predicted to be higher than the value of 0.6 obtained. The MDA-MB-231 cells were used as a negative control and did not demonstrate erbB-2 mediated gene transfer with the Fab-erbB-2 conjugate. Of course, differences in integrin expression between the cell lines may also account for some of these observations. Therefore, analysis to determine the relative expression of integrins on these cell lines will be performed.

Comparisons between targeting of AdCMVLuc on a particular cell line with different conjugates can be made. The Fab-erbB-2 conjugate has a larger Ti than the

Fab-EGF conjugate on BT-474 cells, in good agreement with the scatchard analysis. The Fab-FGF2 has the highest Ti of all in the BT-474 conjugates; however, a scatchard analysis needs to be performed to compare the results to the other two conjugates. The Ti in MDA-MB-468 cells is 11.5 with Fab-EGF and 3.7 with Fab-FGF2, which indicates that there should be more EGF receptors present than FGF receptors. Again, an FGF scatchard analysis on this cell line needs to be performed to determine if this is true. This same comparison can also be made for the MDA-MB-231 cells in which more FGF receptors would be expected than EGF receptors based on the relative Ti values. The SK-BR-3 cells have similar Ti values when targeting with either Fab-EGF or Fab-erbB-2. Scatchard analysis will determine if the number of EGF and erbB-2 receptors is comparable on this cell line.

This report demonstrates that most of the studies proposed to be completed in the Statement of Work by the end of the second year have been completed by the end of the first year. Some additional work needs to completed with regard to binding assays, scatchard analyses, and targeting assays. We will begin to perform targeting assays *in vivo* once the *in vitro* assays are complete. As of now, the BT-474 and MDA-MB-468 cells will be used *in vivo* due to their ability to form tumors (21, 22, 23). The Fab-FGF2 and Fab-erbB-2 conjugates will be used with the BT-474 cells and the Fab-FGF2 and Fab-EGF conjugates will be used with the MDA-MB-468 cells. In the Statement of Work, the *in vivo* experiments were to use adenovirus vectors encoding the firefly luciferase and β-galactosidase reporter genes. In addition to these, an adenovirus vector encoding for the cytosine deaminase (CD) gene will be used to perform therapeutic studies. The CD enzyme converts the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a radiosensitizing and chemotherapeutic agent. We have experience using an adenovirus vector encoding for this gene (AdCMVCD) (24) and believe it will demonstrate specific and enhanced therapeutic utility when targeted to metastatic breast cancer.

Conclusions

This work shows that adenoviral vectors can be targeted to tumor-specific receptors which can result in an increase in gene transfer. Also, there appears to be a correlation between gene transfer and the expression of adenoviral receptor or target receptor on the tumor cell. Additional studies need to be performed to confirm this hypothesis. The appropriate conjugates will be investigated further *in vivo* utilizing adenoviral vectors encoding for either reporter genes or a therapeutic gene (CD). These targeted vectors should have applications in treating patients with brain metastases for reducing neural toxicity and possibly enhancing therapeutic efficacy. Also, "cocktails" of the targeted adenoviral vectors may be useful in bone marrow purging of breast cancer cells that are normally refractory towards adenoviral infection or breast cancer cells of differing phenotypes that respond to one particular targeting moiety.

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Table 1. Scatchard assays on breast cancer cell lines to quantitate the number of FGF, EGF, and erbB-2 receptors per cell.

Cell Line	# FGF receptors/cell	# EGF receptors/cell	# erbB-2 receptors/cell
BT-474	TBP[a]	8.3×10^3	6.6 x 105
MB-468	TBP	7.5×10^5	NA
MB-134	TBP	NA[b]	NA
MB-231	TBP	4.0×10^4	NA
MB-453	TBP	NA	TBP
SK-BR-3	TBP	TBP	TBP

[a]To be performed
[b]Not applicable

Table 2. Summary of binding, scatchard, and targeting data.

Cell Line	%^{125}I-Ad[a]	%^{125}I-FGF2[a]	%^{125}I-EGF[a]	%^{125}I-erbB-2[a]	# receptors/cell[b]	Ti[c] FGF2	Ti EGF	Ti erbB-2
BT-474	8.5	13.3	10.3	44.6	8.3 x 10^3 EGFr 6.6 x 10^5 erbB-2	3.3	0.3	1.3
MB-468	3.9	11.3	46.3	0.0	7.5 x 10^5 EGFr	3.7	11.5	NA
MB-134	4.6	28.1	1.1	1.7	NA[e]	44.6	0.2	NA
MB-231	5.0	19.9	9.0	3.8	4.0 x 10^4 EGFr	4.8	1.3	0.1
MB-453	TBP[d]	24.9	1.3	21.4	erbB-2 TBP	TBP	NA	0.6
SK-BR-3	TBP	8.7	63.4	47.3	EGFr TBP erbB-2 TBP	TBP	1.9	1.6

[a]Represents specific binding of radioligand
[b]FGF2 scatchard will be performed on all cell lines
[c]Targeting index = targeted luciferase expression/non-targeted luciferase expression
[d]To be performed
[e]Not applicable

Figure Legend

Figure 1. Binding of ^{125}I-Ad to BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 breast cancer cells. The cells (1 x 10^6) were incubated with 1 x10^5 cpm of ^{125}I-Ad in the presence or absence of an excess of unlabeled Ad5 knob for 1 h at 4°C. The cells were then washed, centrifuged and counted in a gamma counter. The bars represent the mean ± s.d. of ^{125}I-Ad bound to the cells as a percentage of the total radioactivity added ($n \geq 4$).

Figure 2. Binding of ^{125}I-FGF2 to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. The cells (1 x 10^6) were incubated with 0.05 nM of ^{125}I-FGF2 in the presence or absence of an excess of unlabeled FGF2 for 1 h at 4°C. The cells were then washed, centrifuged and counted in a gamma counter. The bars represent the mean ± s.d. of ^{125}I-FGF2 bound to the cells as a percentage of the total radioactivity added ($n \geq 3$).

Figure 3. Binding of ^{125}I-EGF to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. The cells (1 x 10^6) were incubated with 0.05 nM of ^{125}I-EGF in the presence or absence of an excess of unlabeled EGF for 1 h at 4°C. The cells were then washed, centrifuged and counted in a gamma counter. The bars represent the mean ± s.d. of ^{125}I-EGF bound to the cells as a percentage of the total radioactivity added ($n \geq 3$).

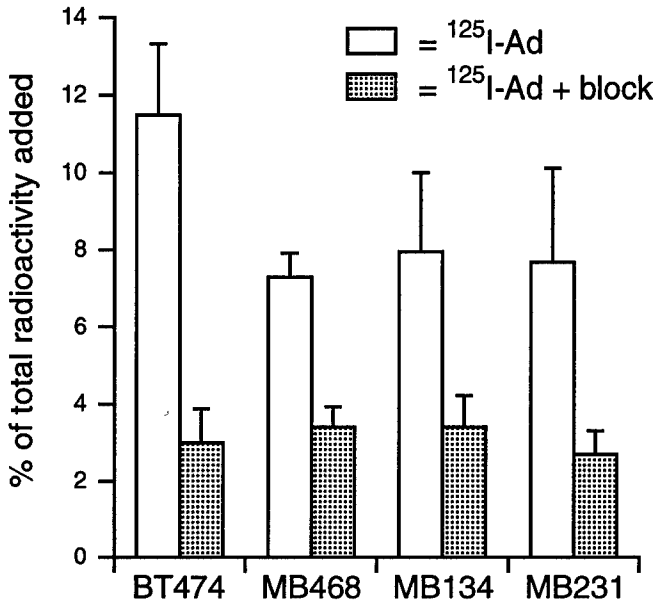
Figure 4. Binding of anti-^{125}I-erbB-2 monoclonal antibody to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. The cells (1 x 10^6) were incubated with 0.05 nM of anti-^{125}I-erbB-2 antibody in the presence or absence of an excess of unlabeled anti-erbB-2 antibody for 1 h at 4°C. The cells were then washed, centrifuged and counted in a gamma counter. The bars represent the mean ± s.d. of ^{125}I-erbB-2 bound to the cells as a percentage of the total radioactivity added ($n \geq 3$).

Figure 5. Targeting of AdCMVLuc (Ad) to the BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 breast cancer cells using the Fab-FGF2 conjugate. The cells (4.8 x 10^4) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-FGF2 (52 ng per 9.6 x 10^5 pfu). A neutralizing amount of Fab or a rabbit anti-FGF2 polyclonal antibody was directly incubated with AdCMVLuc or AdCMVLuc-Fab-FGF2 conjugate, respectively, for 30 min at room temperature prior to infection for blocking gene transfer. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) ($n = 3$).

Figure 6. Targeting of AdCMVLuc (Ad) to the BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, and SK-BR-3 breast cancer cells using the Fab-EGF conjugate. The cells (4.8 x 10^4) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-EGF (13 ng per 9.6 x 10^5 pfu). Cells were blocked with 300 µl/well of

recombinant Ad5 knob (20 μg/ml) or EGF (6.7 μg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate respectively. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) (n = 3).

Figure 7. Targeting of AdCMVLuc (Ad) to the BT-474, MDA-MB-231, MDA-MB-453 and SK-BR-3 breast cancer cells using the Fab-erbB-2 conjugate. The cells (4.8 x 10^4) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-erbB-2 (46 ng per 9.6 x 10^5 pfu). Cells were blocked with 300 μl/well of recombinant Ad5 knob (20 μg/ml) or erbB-2 antibody (10 μg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate respectively. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) (n = 3 for BT-474; n = 6 for other cell lines).


% of total radioactivity added
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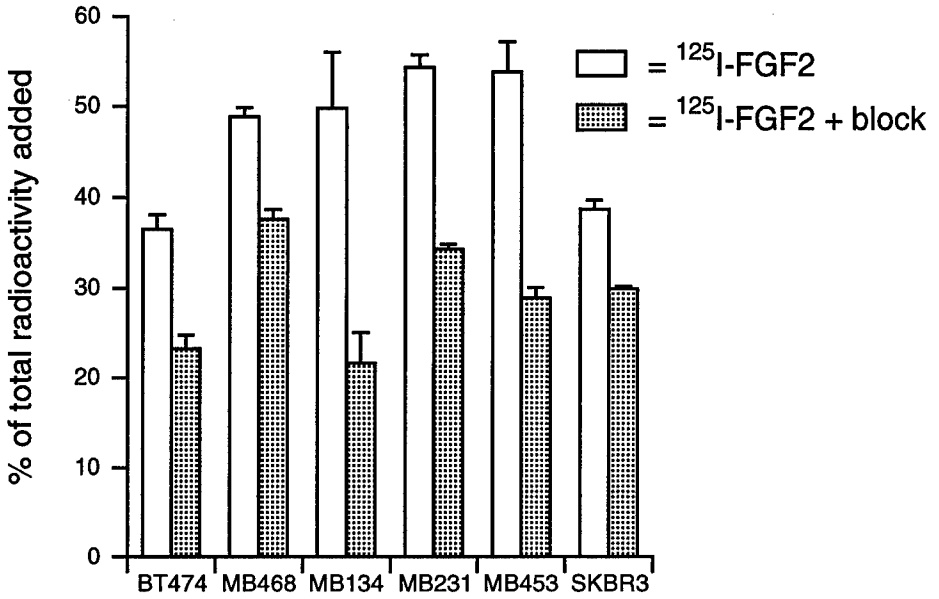
12
10
8
6
4
2
0
= 125I-Ad
= 125I-Ad + block
BT474
MB468
MB134
MB231


% of total radioactivity added
60
50
40
30
20
10
0
BT474
MB468
MB134
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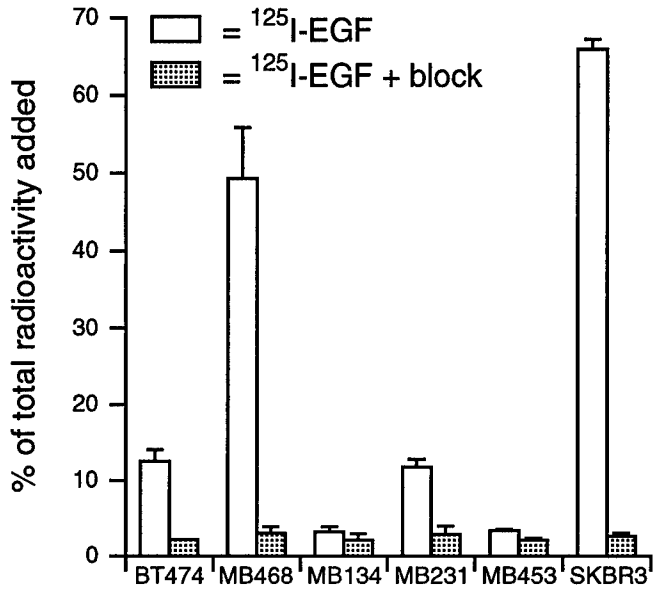
MB453
SKBR3
= 125I-FGF2
= 125I-FGF2 + block


70
60
50
40
30
20
10
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% of total radioactivity added
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= 125I-EGF + block
BT474
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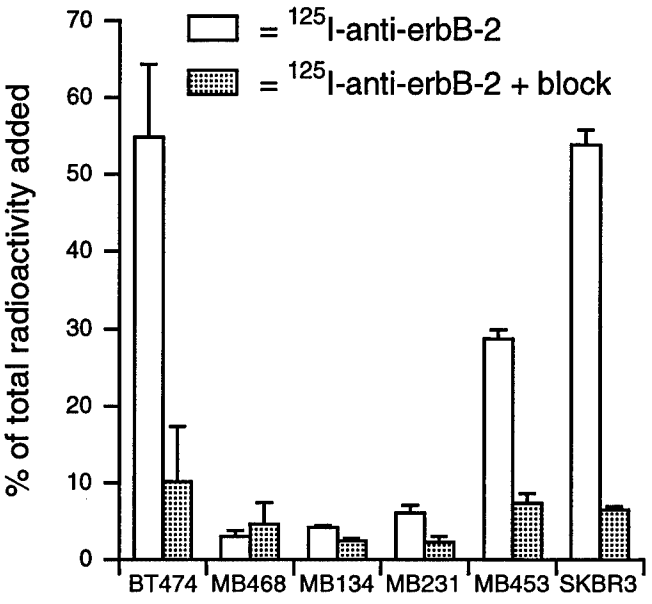
MB134
MB231
MB453
SKBR3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60
50
40
30
20
10
0
= 125I-anti-erbB-2
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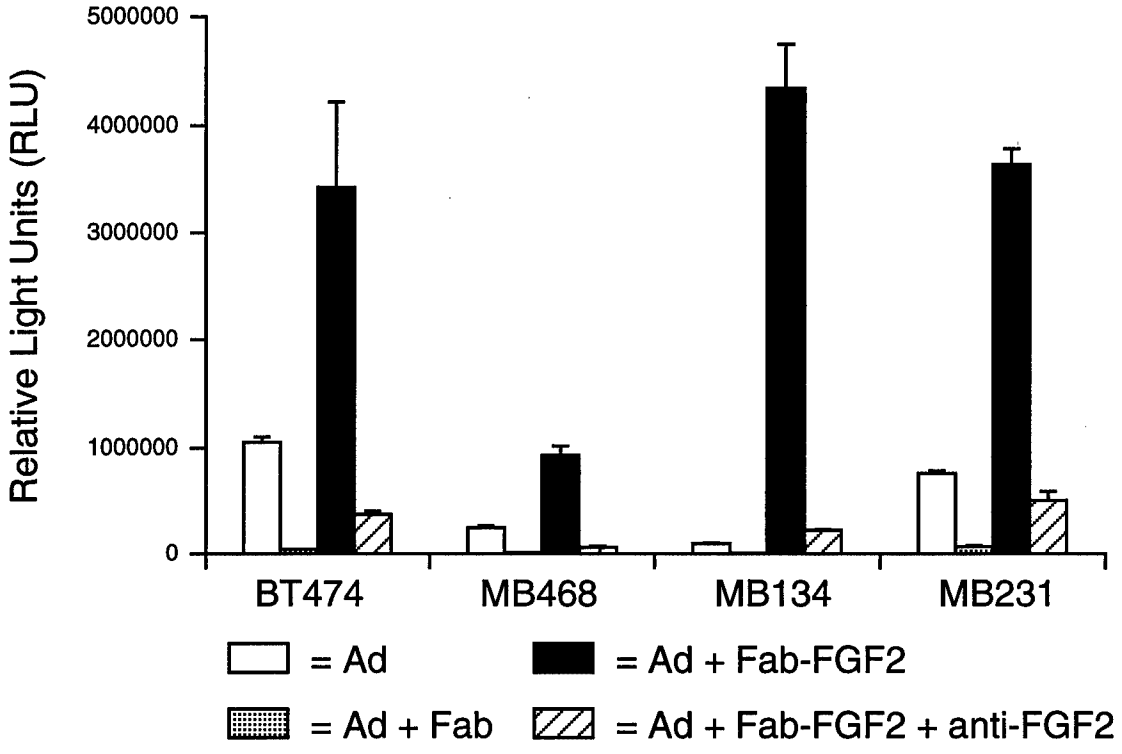
BT474
MB468
MB134
MB231
MB453
SKBR3


Relative Light Units (RLU)
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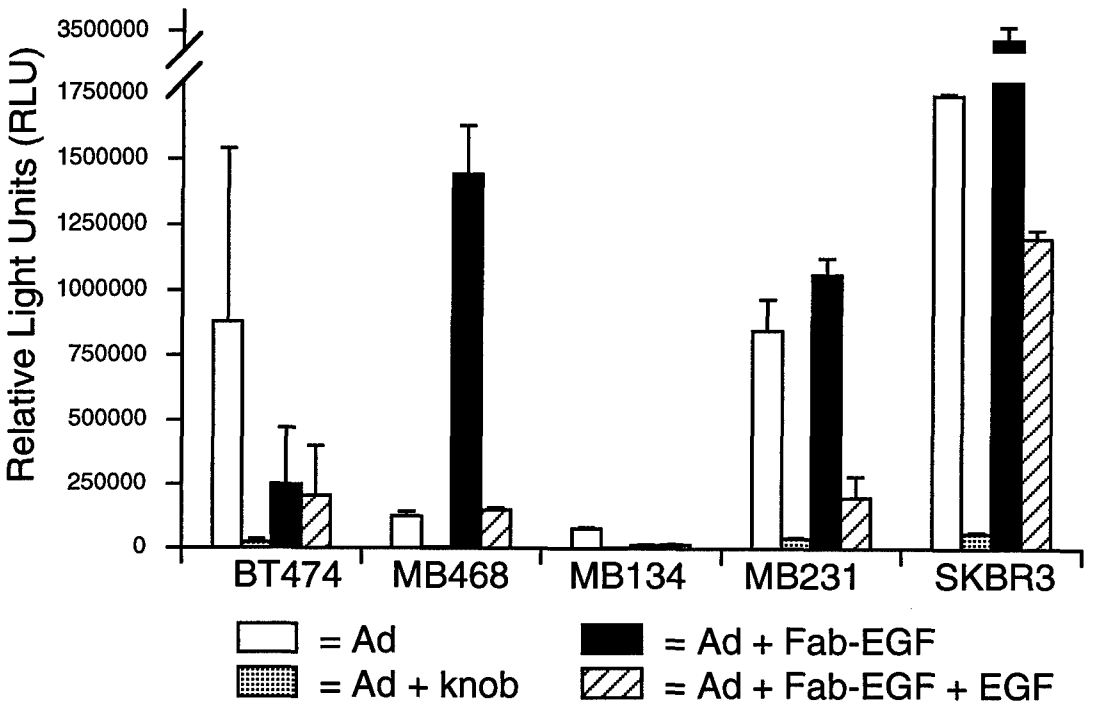
4000000
3000000
2000000
1000000
0
BT474
MB468
MB134
MB231
= Ad
= Ad + Fab-FGF2
= Ad + Fab
= Ad + Fab-FGF2 + anti-FGF2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1750000
1500000
1250000
1000000
750000
500000
250000
0
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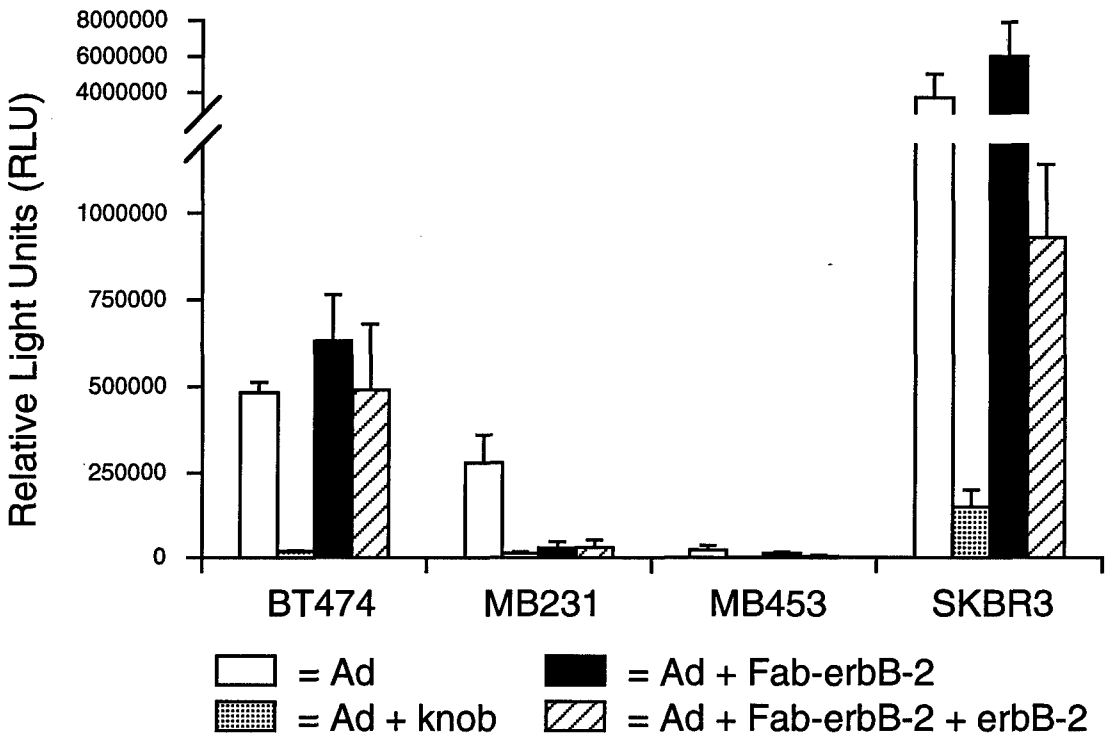
MB468
MB134
MB231
SKBR3
= Ad
= Ad + knob
= Ad + Fab-EGF
= Ad + Fab-EGF + EGF


Relative Light Units (RLU)
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6000000
4000000
1000000
750000
500000
250000
0
BT474
MB231
MB453
SKBR3
= Ad
= Ad + Fab-erbB-2
= Ad + knob
= Ad + Fab-erbB-2 + erbB-2

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